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A TUMOR NECROSIS FACTOR RELATED LIGAND

The present invention relates to Tumor Necrosis Factor Related ligand or "TRELL", a polypeptide which is a member of the Tumor Necrosis Factor Family. The protein or its receptor may have anti-cancer and/or immunoregulatory applications. Furthermore, cells transfected with the gene for TRELL may be used in gene therapy to treat tumors, autoimmune and inflammatory diseases or inherited genetic disorders.

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BACKGROUND OF THE INVENTION

The tumor-necrosis factor (TNF)-related cytokines are mediators of host defense and immune regulation. Members of this family exist in membrane-anchored forms, acting locally through cell-to-cell contact, or as secreted proteins capable of diffusing to more distant targets. A parallel family of receptors signals the presence of these molecules leading to the initiation of cell death or cellular proliferation and differentiation in the target tissue. Presently, the TNF family of ligands and receptors has at least 9 recognized receptor-ligand pairs, including: TNF:TNF-R; LT- α :TNF-R; LT- α/β :LT- β -R; FasL:Fas; CD40L:CD40; CD30L:CD30; CD27L:CD27; OX40L:OX40 and 4-1BBL:4-1BB. The DNA sequences encoding these ligands have only about 25% to about 30% identity in even the most related cases, although the amino acid relatedness is about 50%.

The defining feature of this family of cytokine receptors is found in the cysteine rich extracellular domain initially revealed by the molecular cloning of two distinct TNF receptors.ⁱ This family of genes encodes glycoproteins characteristic of Type I transmembrane proteins with an extracellular ligand binding domain, a single membrane spanning region and a cytoplasmic region involved in activating cellular functions. The cysteine-rich ligand binding region exhibits a tightly knit disulfide linked core domain, which, depending upon the particular family member, is repeated multiple times. Most receptors have four domains, although there may be as few as three, or as many as six.

Proteins in the TNF family of ligands are characterized by a short N-terminal stretch of normally short hydrophilic amino acids, often containing several lysine or

arginine residues thought to serve as stop transfer sequences. Next follows a transmembrane region and an extracellular region of variable length, that separates the C-terminal receptor binding domain from the membrane. This region is sometimes referred to as the "stalk". The C-terminal binding region comprises the bulk of the protein, and often, but not always, contains glycosylation sites. These genes lack the classic signal sequences characteristic of type I membrane proteins, having type II membrane proteins with the C terminus lying outside the cell, and the short N-terminus residing in the cytoplasm. In some cases, e.g., TNF and LT- α , cleavage in the stalk region can occur early during protein processing and the ligand is then found primarily in secreted form. Most ligands, however, exist in a membrane form, mediating localized signalling.

The structure of these ligands has been well-defined by crystallographic analyses of TNF, LT- α , and CD40L. TNF and lymphotoxin- α (LT- α) are both structured into a sandwich of two anti-parallel β -pleated sheets with the "jelly roll" or Greek key topology.ⁱⁱ The rms deviation between the C α and β -strand residues is 0.61 C, suggesting a high degree of similarity in their molecular topography. A structural feature emerging from molecular studies of CD40L, TNF and LT- α is the propensity to assemble into oligomeric complexes. Intrinsic to the oligomeric structure is the formation of the receptor binding site at the junction between the neighboring subunits creating a multivalent ligand. The quaternary structures of TNF, CD40L and LT- α have been shown to exist as trimers by analysis of their crystal structures. Many of the amino acids conserved between the different ligands are in stretches of the scaffold β -sheet. It is likely that the basic sandwich structure is preserved in all of these molecules, since portions of these scaffold sequences are conserved across the various family members. The quaternary structure may also be maintained since the subunit conformation is likely to remain similar.

TNF family members can best be described as master switches in the immune system controlling both cell survival and differentiation. Only TNF and LT α are currently recognized as secreted cytokines contrasting with the other predominantly membrane anchored members of the TNF family. While a membrane form of TNF has been well-characterized and is likely to have unique biological roles, secreted TNF functions as a general alarm signaling to cells more distant from the site of the triggering event. Thus TNF secretion can amplify an event leading to the well-described changes in the vasculature lining and the inflammatory state of cells. In

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contrast, the membrane bound members of the family send signals through the TNF type receptors only to cells in direct contact. For example T cells provide CD40 mediated "help" only to those B cells brought into direct contact via cognate TCR interactions. Similar cell-cell contact limitations on the ability to induce cell death apply to the well-studied Fas system.

The ability to induce programmed cell death is an important and well-studied feature of several members of the TNF family. Fas mediated apoptosis appears to play a role in the regulation of autoreactive lymphocytes in the periphery and possibly the thymus (Castro et al., 1996) and recent work has also implicated the TNF and CD30 systems in the survival of T cells and large cell anaplastic lymphoma lines (Amakawa et al., 1996; Gruss et al., 1994; Sytwu et al., 1996; Zheng et al., 1995). We and others had previously shown the death of this line in response to TNF, Fas or LT β receptor signaling to have features of apoptosis (Abreu-Martin et al., 1995; Browning et al., 1996).

It appears that one can segregate the TNF ligands into three groups based on their ability to induce cell death (Table III). First, TNF, Fas ligand and TRAIL can efficiently induce cell death in many lines and their receptors most likely have good canonical death domains. Presumably the ligand to DR-3 (TRAMP/WSL-1) would also ^{fall} into this category. Next there are those ligands which trigger a weaker death signal limited to few cell types and TRELL, CD30 ligand and LT α 1 β 2 are examples of this class. How this group can trigger cell death in the absence of a canonical death domain is an interesting question and suggests that a separate weaker death signaling mechanism exists. Lastly, there ^{are} those members that cannot efficiently deliver a death signal. Probably all groups can have antiproliferative effects on some cell types consequent to inducing cell differentiation e.g. CD40 (Funakoshi et al., 1994)

The TNF family has grown dramatically in recent years to encompass at least 11 different signaling pathways involving regulation of the immune system. The expression patterns of TRELL and TRAIL indicate that there is still more functional variety to be uncovered in this family. This aspect has been especially highlighted in recent the discovery of two receptors that affect the ability of ^R ~~ous~~ ^{sarcoma} ~~sarcoma~~ and herpes simplex virus to replicate as well as the historical observations that TNF has anti-viral activity and pox viruses encode for decoy TNF receptors (Brojatsch et al., 1996; Montgomery et al., 1996; Smith, 1994; Vassalli, 1992). The generation soluble TRELL

and the identification of the TRELL receptor should provide the tools to elucidate the biological function of this interesting protein.

5 TNF is a mediator of septic shock and cachexiaⁱⁱⁱ, and is involved in the regulation of hematopoietic cell development.^{iv} It appears to play a major role as a mediator of inflammation and defense against bacterial, viral and parasitic infections^v as well as having antitumor activity.^{vi} TNF is also involved in different autoimmune diseases.^{vii} TNF may be produced by several types of cells, including macrophages, fibroblasts, T cells and natural killer cells.^{viii} TNF binds to two different receptors, each acting through specific intracellular signaling molecules, thus resulting in different effects of TNF.^{ix} TNF can exist either as a membrane bound form or as a soluble secreted cytokine.^x

15 LT- α shares many activities with TNF, i.e. binding to the TNF receptors,^{xi} but unlike TNF, appears to be secreted primarily by activated T cells and some β -lymphoblastoid tumors.^{xii} The heteromeric complex of LT- α and LT- β is a membrane bound complex which binds to the LT- β receptor.^{xiii} The LT system (LTs and LT-R) appears to be involved in the development of peripheral lymphoid organs since genetic disruption of LT- β leads to disorganization of T and B cells in the spleen and an absence of lymph nodes.^{xiv} The LT- β system is also involved in cell death of some adenocarcinoma cell lines.^{xv}

20 Fas-L, another member of the TNF family, is expressed predominantly on activated T cells.^{xvi} It induces the death of cells bearing its receptor, including tumor cells and HIV-infected cells, by a mechanism known as programmed cell death or apoptosis.^{xvii} Furthermore, deficiencies in either Fas or Fas-L may lead to lymphoproliferative disorders, confirming the role of the Fas system in the regulation of immune responses.^{xviii} The Fas system is also involved in liver damage resulting from hepatitis chronic infection^{xix} and in autoimmunity in HIV-infected patients.^{xx} The Fas system is also involved in T-cell destruction in HIV patients.^{xxi} TRAIL, another member of this family, also seems to be involved in the death of a wide variety of transformed cell lines of diverse origin.^{xxii}

30 CD40-L, another member of the TNF family, is expressed on T cells and induces the regulation of CD40-bearing B cells.^{xxiii} Furthermore, alterations in the CD40-L gene result in a disease known as X-linked hyper-IgM syndrome.^{xxiv} The CD40 system is also involved in different autoimmune diseases^{xxv} and CD40-L is known to have antiviral properties.^{xxvi} Although the CD40 system is involved in the

rescue of apoptotic B cells,^{xxvii} in non-immune cells it induces apoptosis^{xxviii}. Many additional lymphocyte members of the TNF family are also involved in costimulation.^{xxix}

Generally, the members of the TNF family have fundamental regulatory roles in controlling the immune system and activating acute host defense systems. Given the current progress in manipulating members of the TNF family for therapeutic benefit, it is likely that members of this family may provide unique means to control disease. Some of the ligands of this family can directly induce the apoptotic death of many transformed cells eg. LT, TNF, Fas ligand and TRAIL (Nagata, 1997). Fas and possibly TNF and CD30 receptor activation can induce cell death in nontransformed lymphocytes which may play an immunoregulatory function (Amakawa et al., 1996; Nagata, 1997; Sytwu et al., 1996; Zheng et al., 1995). In general, death is triggered following the aggregation of death domains which reside on the cytoplasmic side of the TNF receptors. The death domain orchestrates the assembly of various signal transduction components which result in the activation of the caspase cascade (Nagata, 1997). Some receptors lack canonical death domains, e.g. LT β receptor and CD30 (Browning et al., 1996; Lee et al., 1996) yet can induce cell death, albeit more weakly. It is likely that these receptors function primarily to induce cell differentiation and the death is an aberrant consequence in some transformed cell lines, although this picture is unclear as studies on the CD30 null mouse suggest a death role in negative selection in the thymus (Amakawa et al., 1996). Conversely, signaling through other pathways such as CD40 is required to maintain cell survival. Thus, there is a need to identify and characterize additional molecules which are members of the TNF family thereby providing additional means of controlling disease and manipulating the immune system.

SUMMARY OF THE INVENTION

Accordingly, the present invention is directed to a polypeptide, a tumor necrosis factor related ligand called TRELL which substantially obviates one or more of the problems due to the limitations and disadvantages of the related art. The inventor has discovered a new member of the TNF family of cytokines, and defined both the human and murine amino acid sequence of the protein, as well as the DNA sequences encoding these protein. The claimed invention may be used to identify new diagnostics and therapeutics for numerous diseases and conditions as discussed in more detail below, as well as to obtain information about, and manipulate, the immune system and

its processes. Additionally, the claimed invention is involved in the induction of cell death in carcinoma.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent ^{from} ~~form~~ the description, or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained by the compositions and methods particularly pointed out in the written description and claims hereof, as well as in the appended drawings.

Thus, to achieve these and other advantages, and in accordance with the purpose of the invention, as embodied and broadly described herein, the invention includes a DNA sequence encoding TRELL. The nucleotide sequence for mouse TRELL (mTRELL) is shown in SEQ ID. NO. 1, and for human TRELL (hTRELL) in SEQ ID. NO. 3. Specifically, the invention relates to DNA sequences which encode a TRELL having the amino acid sequence identified in SEQ. ID. NO. 2 (mTRELL) or 4 (hTRELL). In other embodiments, the invention relates to sequences that have at least 50% homology with the DNA encoding the C terminal receptor binding domain of TRELL and hybridize to the claimed DNA sequences or fragments thereof, and which encode TRELL having the sequence identified in SEQ. ID. NO. 4 or SEQ ID. NO. 2.

The invention in certain embodiments furthermore relates to a DNA sequence encoding TRELL where the sequence is operatively linked to an expression control sequence. Any suitable expression control sequence is useful in the claimed invention, and can easily be selected by one skilled in the art.

The invention also contemplates a recombinant DNA comprising a sequence encoding TRELL, or a fragment thereof, as well as hosts with stably integrated TRELL sequences introduced into their genome, or possessing episomal elements. Any suitable host may be used in the invention, and can easily be selected by one skilled in the art without undue experimentation.

In other embodiments, the invention relates to methods of producing substantially pure TRELL comprising the step of culturing transformed hosts, and
30 TRELL essentially free of normally associated animal proteins.

The invention encompasses TRELL having the amino acid sequence identified in SEQ. ID. NO. 4 or SEQ ID. NO. 2 as well as fragments or homologs thereof. In various embodiments, the amino acid and/or the DNA sequence of TRELL may

comprise conservative insertions, deletions and substitutions, as further defined below or may comprise fragments of said sequences.

The invention relates in other embodiments to soluble TRELL constructs, which may be used to directly trigger TRELL mediated pharmacological events. Such events
5 may have useful therapeutic benefit in the treatment of cancer or the manipulation of the immune system to treat immunologic diseases. Soluble TRELL forms could be genetically reengineered to incorporate an easily recognizable tag, thereby facilitating the identification of TRELL receptors.

In yet other embodiments the invention relates to methods of gene therapy using
10 the TRELL's disclosed and claimed herein.

The pharmaceutical preparations of the invention may, optionally, include pharmaceutically acceptable carriers, adjuvants, fillers, or other pharmaceutical compositions, and may be administered in any of the numerous forms or routes known in the art.

15 It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory, and are intended to provide further explanation of the invention as claimed.

The accompanying drawings are included to provide a further understanding of the invention, and are incorporated in, and constitute a part of this specification,
20 illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an amino acid sequence comparison of human and mouse TRELL. (SEQ ID NO: 4) (SEQ ID NO: 2)

25 Figures 2A and 2B are an amino acid comparison of human members of the
TNF family.

Figure 3 is a northern analysis of TRELL mRNA expression in different mouse cell lines and tissues. Lanes are duplicated and contained RNA from (1) thioglycolate induced peritoneal macrophages, (2) bone marrow, (3) spleen, and (4) liver.

30 Figure 4 is a northern analysis of TRELL mRNA expression in different human tissues.

Figure 5: SDS-PAGE of recombinant TNF, LT α and TRELL under reducing and nonreducing conditions. (designated here as TWEAK)

Figure 6: TRELL is cytotoxic to the human adenocarcinoma line HT29.

- 5 A. Ability of the TNF, TRELL, LT α / β and anti-Fas to block the growth of the HT29 line in the presence of human interferon-g. Cells were grown for 4 days in the presence of the various agents and growth was assessed using MTT staining.
- 10 B. Morphology of the cells undergoing cell death. Cells were pregrown for 2 days and then treated for 24 hours with 80 U/ml ^{interferon-g with} ~~interferon-g and A. no~~ further addition. ~~B. 100 ng/ml fixed with ethanol and shown by phase contrast microscopy in the top panels (400x magnification) and stained with 1 ug/ml Hoescht dye to reveal the nuclei in the bottom panel. Typical cells with condensed nuclei characteristic of apoptosis are indicated with arrows.~~

DETAILED DESCRIPTION

Reference will now be made in detail to the present preferred embodiments of the invention. This invention relates to DNA sequences that code for human or mouse TRELL, fragments and homologs thereof, and expression of those DNA sequences in hosts transformed with them. The invention relates to uses of these DNA sequences and the peptides encoded by them. Additionally, the invention encompasses both human and mouse amino acid sequences for TRELL, or fragments thereof, as well as pharmaceutical compositions comprising or derived from them.

A. DEFINITIONS

"Homologous", as used herein, refers to the sequence similarity between sequences of molecules being compared. When a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that position. The percent of homology between two sequences is a function of the number of matching or homologous positions shared by the two sequences divided by the number of positions compared x 100. For example, if 6 of 10, of the positions in two sequences are matched or homologous then the two sequences are 60% homologous. By way of example, the DNA sequences ATTGCC and TATGGC share 50% homology. Generally, a comparison is made when two sequences are aligned to give maximum homology.

A "purified preparation" or a "substantially pure preparation" of a polypeptide, as used herein, means a polypeptide that has been separated from other proteins, lipids, and nucleic acids with which it naturally occurs. Preferably, the polypeptide is also

separated from other substances, e.g., antibodies, matrices, etc., which are used to purify it.

“Transformed host” as used herein is meant to encompass any host with stably integrated sequence, i.e. TRELL sequence, introduced into its genome or a host
5 possessing sequence, i.e. TRELL, encoding episomal elements.

A “treatment”, as used herein, includes any therapeutic treatment, e.g., the administration of a therapeutic agent or substance, e.g., a drug.

A “substantially pure nucleic acid”, e.g., a substantially pure DNA, is a nucleic acid which is one or both of: not immediately contiguous with either one or both of the
10 sequences, e.g., coding sequences, with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the nucleic acid is derived; or (2) which is substantially free of a nucleic acid sequence with which it occurs in the organism from which the nucleic acid is derived. The term includes, for example, a recombinant DNA which is incorporated
15 into a vector, e.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other DNA sequences. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding
20 TRELL.

The terms “peptides”, “proteins”, and “polypeptides” are used interchangeably herein.

“Biologically active” as used herein, means having an in vivo or in vitro activity which may be performed directly or indirectly. Biologically active fragments of
25 TRELL may have, for example, 70% amino acid homology with the active site of TRELL, more preferably at least 80%, and most preferably, at least 90% homology. Identity or homology with respect to TRELL is defined herein as the percentage of amino acid residues in the candidate sequence which are identical to the TRELL residues in SEQ. ID. NOS. 2 or 4.

30 The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature.^{xxx}

B. DNA SEQUENCES OF THE INVENTION

As described herein, one aspect of the invention features a substantially pure (or recombinant) nucleic acid which includes a nucleotide sequence encoding a TRELL polypeptide, such as the DNA described in SEQ. ID. NO. 1 or 3 and/or equivalents of such nucleic acids. The term nucleic acid as used herein can include fragments and equivalents, such as, for example, sequences encoding functionally equivalent peptides. Equivalent nucleotide sequences may include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants, mutations, etc. and include sequences that differ from the nucleotide sequence encoding TRELL shown in SEQ ID NO:1 or 3, due to the degeneracy of the genetic code. The inventors have sequenced a human 1936 bp DNA which contains an open reading frame encoding a TRELL polypeptide, having the 248 amino acid sequence as identified in SEQ. ID. NO. 4.

The inventor describes herein both human and murine sequences; the invention will be described generally by reference to the human sequences, although one skilled in the art will understand that the mouse sequences are encompassed herein. A striking feature of TRELL is the extensive sequence conservation of the receptor binding domain between mouse and man; only the Fas ligand approaches this level of conservation. Both the murine and human TRELL proteins have all of the characteristics of the TNF family, i.e., a type II membrane protein organization and conservation of the sequence motifs involved in the folding of the protein into the TNF ant-parallel β -sheet structure.

The nucleotide sequence for mTRELL is set forth in SEQ. ID. NO. 1; the amino acid sequence for mTRELL is described in SEQ. ID. NO. 2. The DNA and amino acid sequences for hTRELL are described in SEQ. ID. NOS. 3 and 4 respectively.

The sequences of the invention can be used to prepare a series of DNA probes that are useful in screening various collections of natural and synthetic DNAs for the presence of DNA sequences that code for TRELL or fragments or derivatives thereof. One skilled in the art will recognize that reference to "TRELL", as used herein, refers also to biologically active derivatives, fragments or homologs thereof.

The DNA sequences encoding TRELL of the invention can be employed to produce TRELL peptides on expression in various prokaryotic and eukaryotic hosts transformed with them. These TRELL peptides may be used in anti-cancer, and immunoregulatory applications. In general, this comprises the steps of culturing a host

transformed with a DNA molecule containing the sequence encoding TRELL, operatively-linked to an expression control sequence.

The DNA sequences and recombinant DNA molecules of the present invention can be expressed using a wide variety of host/vector combinations. For example, 5 useful vectors may consist of segments of chromosomal, non-chromosomal or synthetic DNA sequences. The expression vectors of the invention are characterized by at least one expression control sequence that may be operatively linked to a TRELL DNA sequence inserted in the vector, in order to control and to regulate the expression of the DNA sequence.

10 Furthermore, within each expression vector, various sites may be selected for insertion of a TRELL sequence of the invention. The sites are usually designated by a restriction endonuclease which cuts them, and these sites and endonucleases are well recognized by those skilled in the art. It is of course to be understood that an expression vector useful in this invention need not have a restriction endonuclease site 15 for insertion of the desired DNA fragment. Instead, the vector may be cloned to the fragment by alternate means. The expression vector, and in particular the site chosen therein for insertion of a selected DNA fragment, and its operative linking therein to an expression control sequence, is determined by a variety of factors. These factors include, but are not limited to, the size of the protein to be expressed, the susceptibility 20 of the desired protein to proteolytic degradation by host cell enzymes, number of sites susceptible to a particular restriction enzyme, contamination or binding of the protein to be expressed by host cell proteins which may prove difficult to remove during purification. Additional factors which may be considered include expression characteristics such as the location of start and stop codons relative to the vector 25 sequences, and other factors which will be recognized by those skilled in the art. The choice of a vector and insertion site for the claimed DNA sequences is determined by a balancing of these factors, not all selections being equally effective for a desired application. However, it is routine for one skilled in the art to analyze these parameters and choose an appropriate system depending on the particular application.

30 One skilled in the art can readily make appropriate modifications to the expression control sequences to obtain higher levels of protein expression, i.e. by substitution of codons, or selecting codons for particular amino acids that are preferentially used by particular organisms, to minimize proteolysis or to alter

glycosylation composition. Likewise, cysteines may be changed to other amino acids to simplify production, refolding or stability problems.

Thus, not all host/expression vector combinations function with equal efficiency in expressing the DNA sequences of this invention. However, a particular selection of a host/expression vector combination may be made by those of skill in the art. Factors one may consider include, for example, the compatibility of the host and vector, toxicity to the host of the proteins encoded by the DNA sequence, ease of recovery of the desired protein, expression characteristics of the DNA sequences and expression control sequences operatively linked to them, biosafety, costs and the folding, form or other necessary post-expression modifications of the desired protein.

The TRELL and homologs thereof produced by hosts transformed with the sequences of the invention, as well as native TRELL purified by the processes of this invention, or produced from the claimed amino acid sequences, are useful in a variety of compositions and methods for anticancer and immunoregulatory applications. They are also useful in therapy and methods directed to other diseases.

This invention also relates to the use of the DNA sequences disclosed herein to express TRELL under abnormal conditions, i.e. in a gene therapy setting. TRELL may be expressed in tumor cells under the direction of promoters appropriate for such applications. Such expression could enhance anti-tumor immune responses or directly affect the survival of the tumor. Cytokines such as TRELL can also affect the survival of an organ graft by altering the local immune response. In this case, the graft itself or the surrounding cells would be modified with an engineered TRELL gene.

Another aspect of the invention relates to the use of the isolated nucleic acid encoding TRELL in "antisense" therapy. As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotides or their derivatives which specifically hybridize under cellular conditions with the cellular mRNA and/or DNA encoding TRELL, so as to inhibit expression of the encoded protein, i.e. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to a range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid, which, when transcribed in the cell, produces RNA which is

complementary to at least a portion of the cellular mRNA which encodes TRELL. Alternatively, the antisense construct can be an oligonucleotide probe which is generated ex vivo. Such oligonucleotide probes are preferably modified oligonucleotides which are resistant to endogenous nucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidates, phosphothioate and methylphosphonate analogs of DNA (See, e.g., 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van Der Krol et al., (1988) Biotechniques 6:958-976; and Stein et al. (1988) Cancer Res 48: 2659-2668, specifically incorporated herein by reference.

C. TRELL AND ITS AMINO ACID SEQUENCES

The Tumor Necrosis Factor Family Related Protein (TRELL) of the invention, as discussed above, is a member of the TNF family. The protein, fragments or homologs thereof may have wide therapeutic and diagnostic applications.

TRELL is present in many tissues, in a pattern that is relatively unique among members of the TNF family. Since members of the TNF family are involved in the regulation of a cell death and survival, and cell differentiation, it is possible that TRELL is also involved in cell survival, differentiation, and repair in various tissues.

Although the precise three dimensional structure of TRELL is not known, it is predicted that, as a member of the TNF family, it may share certain structural characteristics with other members of the family. Both mouse and human TRELL are disclosed herein. Mouse TRELL, as deduced from the existing cDNA sequence, comprises a stretch of at least 21 hydrophobic amino acids, which presumably acts as a membrane anchoring domain for a type II membrane protein. The amino acid sequence of mTRELL is described in SEQ ID. NO. 2.

Human TRELL comprises an N-terminal hydrophilic cytoplasmic domain, a roughly 27 amino acid hydrophobic, transmembrane type II domain and a 204 amino acid extracellular domain. The amino acid sequence of hTRELL is described in SEQ. ID. NO. 4.

Figure 1 depicts an amino acid sequence comparison of human and mouse TRELL.

While a 52 amino acid N-terminal region can be predicted from an open reading frame in the cDNA clone, the exact starting methionine cannot be predicted. Met-36 has a reasonable consensus Kozak sequence which may make it the preferred starting

codon. Comparison of the TRELL sequence with other members of the human TNF family reveals considerable structural similarity. For example, as can be seen in Figures 2A and 2B, all the proteins resemble Type II membrane proteins, and share several regions of sequence conservation in the extracellular domain. Regions with bars over the sequences indicate those sequences in TNF and LT α involved in a β strand organization of the molecules. Putative N-linked glycosylation sites are underlined. Asterisks indicate the cysteines involved in a disulfide linkage in TNF. The conserved domains are likely to be involved in intersubunit interactions and sheet organization.

10 An EST search revealed a human clone of 345 bases which is highly homologous to the mouse TRELL. A human TRELL amino acid sequence is set forth in SEQ.ID. NO. 4. The open reading frames encoded by the EST do not contain the sequence motifs which would allow one to characterize this sequence as a member of the TNF family of ligands, e.g. the motif used by Wiley et al. to identify a TRAIL EST
15 within the existing database.

 The novel polypeptides of the invention specifically interact with a receptor, which has not yet been identified. However, the peptides and methods disclosed herein enable the identification of receptors which specifically interact with TRELL or fragments thereof.

20 The claimed invention in certain embodiments includes peptides derived from TRELL which have the ability to bind with TRELL receptors. Fragments of TRELL can be produced in several ways, e.g., recombinantly, by PCR, proteolytic digestion or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end or both ends of a nucleic
25 acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNA's which encode a variety of fragments. DNA's which encode fragments of a protein can also be generated by random shearing, restriction digestion or a combination of the above discussed methods.

30 Polypeptide fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-moc or t-boc chemistry. For example, peptides and DNA sequences of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragment, or divided

into overlapping fragments of a desired length. Methods such as these are described in more detail below.

D. Generation of Soluble TRELL

Soluble forms of the ligand can often signal effectively and hence can be administered as a drug which now mimics the natural membrane form. It is possible that TRELL is naturally secreted as a soluble cytokine, however, if it is not, one can reengineer the gene to force secretion. To create a soluble secreted form of TRELL, one would remove at the DNA level the N-terminus transmembrane regions, and some portion of the stalk region, and replace them with a type I leader or alternatively a type II leader sequence that will allow efficient proteolytic cleavage in the chosen expression system. A skilled artisan could vary the amount of the stalk region retained in the secretion expression construct to optimize both receptor binding properties and secretion efficiency. For example, the constructs containing all possible stalk lengths, i.e. N-terminal truncations, could be prepared such that proteins starting at amino acids 81 to 139 would result. The optimal length stalk sequence would result from this type of analysis.

E. Generation of Antibodies Reactive with TRELL

The invention also includes antibodies specifically reactive with TRELL or its receptor. Anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)). A mammal such as a mouse, a hamster or rabbit can be immunized with an immunogenic form of the peptide. Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers, or other techniques, well known in the art.

An immunogenic portion of TRELL or its receptor can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

In a preferred embodiment, the subject antibodies are immunospecific for antigenic determinants of TRELL or its receptor, e.g. antigenic determinants of a polypeptide of SEQ ID NO: 2 or 4, or a closely related human or non-human mammalian homolog (e.g. 70, 80 or 90 percent homologous, more preferably at least 95 percent homologous). In yet a further preferred embodiment of the present

invention, the anti-TRELL or anti-TRELL-receptor antibodies do not substantially cross react (i.e. react specifically) with a protein which is e.g., less than 80 percent homologous to SEQ ID NO 2 or 4; preferably less than 90 percent homologous with SEQ ID NO: 2 or 4; and, most preferably less than 95 percent homologous with SEQ ID NO: 2 or 4. By "not substantially cross react", it is meant that the antibody has a binding affinity for a non-homologous protein which is less than 10 percent, more preferably less than 5 percent, and even more preferably less than 1 percent, of the binding affinity for a protein of SEQ ID NO 2 or 4.

The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with TRELL or TRELL-receptor. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibodies of the present invention are further intended to include biospecific and chimeric molecules having anti-TRELL or anti-TRELL-receptor activity. Thus, both monoclonal and polyclonal antibodies (Ab) directed against TRELL and its receptor, and antibody fragments such as Fab' and F(ab')₂, can be used to block the action of TRELL and its receptor.

Various forms of antibodies can also be made using standard recombinant DNA techniques. (Winter and Milstein, Nature 349: 293-299 (1991) specifically incorporated by reference herein.) For example, chimeric antibodies can be constructed in which the antigen binding domain from an animal antibody is linked to a human constant domain (e.g. Cabilly et al., U.S. 4,816,567, incorporated herein by reference). Chimeric antibodies may reduce the observed immunogenic responses elicited by animal antibodies when used in human clinical treatments.

In addition, recombinant "humanized antibodies" which recognize TRELL or its receptor can be synthesized. Humanized antibodies are chimeras comprising mostly human IgG sequences into which the regions responsible for specific antigen-binding have been inserted. Animals are immunized with the desired antigen, the corresponding antibodies are isolated, and the portion of the variable region sequences responsible for specific antigen binding are removed. The animal-derived antigen binding regions are then cloned into the appropriate position of human antibody genes in which the antigen binding regions have been deleted. Humanized antibodies

minimize the use of heterologous (i.e. inter species) sequences in human antibodies, and thus are less likely to elicit immune responses in the treated subject.

Construction of different classes of recombinant antibodies can also be accomplished by making chimeric or humanized antibodies comprising variable domains and human constant domains (CH1, CH2, CH3) isolated from different classes of immunoglobulins. For example, antibodies with increased antigen binding site valencies can be recombinantly produced by cloning the antigen binding site into vectors carrying the human : chain constant regions. (Arulanandam et al., J. Exp. Med., 177: 1439-1450 (1993), incorporated herein by reference.)

In addition, standard recombinant DNA techniques can be used to alter the binding affinities of recombinant antibodies with their antigens by altering amino acid residues in the vicinity of the antigen binding sites. The antigen binding affinity of a humanized antibody can be increased by mutagenesis based on molecular modeling. (Queen et al., Proc. Natl. Acad. Sci. 86: 10029-33 (1989) incorporated herein by reference.

F. Generation of Analogs: Production of Altered DNA and Peptide Sequences

Analogues of TRELL can differ from the naturally occurring TRELL in amino acid sequence, or in ways that do not involve sequence, or both. Non-sequence modifications include in vivo or in vitro chemical derivatization of TRELL. Non-sequence modifications include, but are not limited to, changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

Preferred analogs include TRELL or biologically active fragments thereof, whose sequences differ from the sequence given in SEQ ID NOS. 2 and 4, by one or more conservative amino acid substitutions, or by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the activity of TRELL. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g. substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and, phenylalanine, tyrosine.

TABLE 1
CONSERVATIVE AMINO ACID REPLACEMENTS

for amino Acid	code	replace with any of:
Alanine	A	D-Ala, Gly, Beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, -Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met

Lysine	K	D-Lys, Arg, D-Arg, Homo-arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D- Ile, Leu, D-Leu, Val, D- Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L- Dopa, His, D-His, Trp, D- Trp, Trans-3, 4 or 5- phenylproline, cis-3, 4, or 5-phenylproline
Proline	P	D-Pro, L-I-thoazolidine-4- carboxylic acid, D-or L-1- oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo- Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo- Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L- Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D- Ile, Met, D-Met

Useful methods for mutagenesis include PCR mutagenesis and saturation mutagenesis as discussed in more detail below. A library of random amino acid

sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences.

-PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity can be used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized can be amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments can be inserted into appropriate cloning vectors to provide random mutant libraries.

-Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a genetic selection for mutant fragments both neutral substitutions, as well as of a protein can be prepared by random mutagenesis of DNA which those that alter function, can be obtained. The distribution of point mutations is not biased toward conserved sequence elements.

-Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art^{xxxi}. Such techniques have been employed in the directed evolution of other proteins^{xxxii}.

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and

then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

-Alanine Scanning Mutagenesis

5 Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989) specifically incorporated by reference. In alanine scanning, a residue or group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by
10 a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions can then be refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an
15 amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

20 -Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983) incorporated herein by reference. Briefly, the desired DNA can be altered by hybridizing an oligonucleotide encoding a mutation to a DNA template,
25 where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least
30 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that

described by Crea et al. (*Proc. Natl. Acad. Sci. USA*, 75: 5765[1978]) incorporated herein by reference.

-Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34:315[1985]) incorporated herein by reference. The starting material can be a plasmid (or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

-Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. E.g., the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

Various techniques are known in the art for screening generated mutant gene products. Techniques for screening large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the

resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, e.g., in this case, binding to TRELL or its receptor, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

The invention also provides for reduction of the protein binding domains of the subject TRELL polypeptides or their receptors, to generate mimetics, e.g. peptide or non-peptide agents. The peptide mimetics are able to disrupt binding of a TRELL and its receptor. The critical residues of TRELL involved in molecular recognition of a receptor polypeptide or of a downstream intracellular protein, can be determined and used to generate TRELL or its receptor-derived peptidomimetics which competitively or noncompetitively inhibit binding of the TRELL with a receptor/ (see, for example, "Peptide inhibitors of human papilloma virus protein binding to retinoblastoma gene protein" European patent applications EP-412,762A and EP-~~3~~31,080A), specifically incorporated herein by reference.

G. PHARMACEUTICAL COMPOSITIONS

By making available purified and recombinant-TRELL, the present invention provides assays which can be used to screen for drug~~s~~ candidates which are either agonists or antagonists of the normal cellular function, in this case, of TRELL or its receptor. In one embodiment, the assay evaluates the ability of a compound to modulate binding between TRELL and its receptor. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by the skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target

as may be manifest in an alteration of binding affinity with other proteins or change in enzymatic properties of the molecular target.

Pharmaceutical compositions of the invention may comprise a therapeutically effective amount of TRELL or TRELL-receptor, or fragments or mimetics thereof, and, optionally may include pharmaceutically acceptable carriers. Accordingly, this invention provides methods for treatment of cancer, and methods of stimulating, or in certain instances, inhibiting the immune system, or parts thereof by administering a pharmaceutically effective amount of a compound of the invention or its pharmaceutically acceptable salts or derivatives. It should of course be understood that the compositions and methods of this invention can be used in combination with other therapies for various treatments.

The compositions can be formulated for a variety of routes of administration, including systemic, topical or localized administration. For systemic administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the compositions of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compositions may be formulated in solid form and, optionally, redissolved or suspended immediately prior to use. Lyophilized forms are also included in the invention.

The compositions can be administered orally, or by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are known in the art, and include, for example, for transmucosal administration, bile salts, fusidic acid derivatives, and detergents. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the compositions are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the compositions of the invention are formulated into ointments, salves, gels, or creams as known in the art.

Preferably the compositions of the invention will be in the form of a unit dose and will be administered one or more times a day. The amount of active compound administered at one time or over the course of treatment will depend on many factors. For example, the age and size of the subject, the severity and course of the disease being treated, the manner and form of administration, and the judgments of the treating physician. However, an effective dose may be in the range of from about 0.005 to

about 5 mg/kg/day, preferably about 0.05 to about 0.5 mg/kg/day. One skilled in the art will recognize that lower and higher doses may also be useful.

Gene constructs according to the invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a TRELL polypeptide.

Expression constructs of TRELL can be administered in any biologically effective carrier, e.g., any formulation or composition capable of effectively delivering the gene for TRELL to cells in vivo. Approaches include insertion of the gene in viral vectors which can transfect cells directly, or delivering plasmid DNA with the help of, for example, liposomes, or intracellular carriers, as well as direct injection of the gene construct. Viral vector transfer methods are preferred.

A pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA, RNA or amino acid sequences to which they specifically bind. In other aspects, the claimed invention may be used to evaluate a chemical entity for its ability to interact with, e.g., bind or physically associate with a TRELL polypeptide, or fragment thereof. The method includes contacting the chemical entity with the TRELL polypeptide, and evaluating the ability of the entity to interact with the TRELL. Additionally, the TRELL of the invention can be used in methods of evaluating naturally occurring ligands or receptors of TRELL, as well as to evaluate chemical entities which associate or bind with receptors of TRELL.

In certain aspects, the claimed invention features a method for evaluating a chemical entity for the ability to modulate the interaction between TRELL and its receptor. The method includes combining a TRELL receptor, and TRELL under conditions wherein the pair is capable of interacting, adding the chemical entity to be evaluated and detecting the formation or dissolution of complexes. These modulating agents may be further evaluated in vitro, e.g. by testing its activity in a cell free system,

and then, optionally administering the compound to a cell or animal, and evaluating the effect.

H. EXAMPLES

1. ISOLATION OF TRELL cDNAS

5 a) Cloning of murine TRELL

The cDNA coding for mTRELL was isolated by PCR from a cDNA library from mouse peritoneal macrophages. The amino acid sequence and the placement of the transmembrane region are typical of a membrane protein. The amino acid sequence of mTRELL is set forth in SEQ. ID. NO. 2, and the DNA sequence is set forth in SEQ.

10 ID. NO. 1.

Macrophage cells were obtained from Balb/c mice by peritoneal lavage and cells that adhered to plastic within one hour were lysed and processed for RNA extraction. An antisense oligonucleotide primer 5'GTTCCAGGCCAGCCTGGG3' (SEQ ID NO: 5) from a mouse erythropoietin sequence was synthesized. C.B. Shoemaker and L.D. Mistock, "Murine erythropoietin gene: cloning, expression and human gene homology", Mol. Cell. Biol., 6, 849 (1986), specifically incorporated herein by reference. This primer was used in a 5' RACE protocol following the recommendation of the manufacturer (5' RACE system from BRL) in association with the BRL-designed anchor primer. A first strand of cDNA was made from RNA from one hour adherent peritoneal macrophages. Amplification was done in a Perkin Elmer DNA thermal cycler with Taq DNA polymerase from Perkin Elmer. After a denaturation of 5 min. at 94°C, cycling conditions were as follows: 35 cycles at 94°C for 30 sec., 55°C for 30 sec. and 72°C for 3 min. An additional extension at 72°C was performed and then reactions were held at 4°C. Analysis of the PCR experiment on agarose gel revealed 2 amplified fragments of 650bp and 500bp. The 2 fragments were excised from the gel, inserted in pBS-T vectors and sequenced. The two inserts were different. They both had at each extremity the same erythropoietin gene specific oligonucleotide used to prime the PCR synthesis. Northern hybridizations with 32P labeled-random-primed fragments indicated that they hybridized to two different RNA, the 500bp fragment hybridizing to a 1.4 kb RNA in macrophages. 32P-labeled-riboprobes in both directions were used in Northern hybridization to determine the orientation of the cDNA.

From the determined orientations and sequences, two internal primers for the 1.4Kb mRNA were derived. These were used in 3' and 5' RACE-PCR respectively.

3' RACE

The 3' RACE experiment revealed a 750bp fragment which was inserted in a pBS-T vector and sequenced. It corresponds to the 3' end of the 1.4 Kb RNA since the sequence possess a polyA addition signal AATAAA just prior to the poly A tract. The 5' RACE did not reveal any band. The Clontech Marathon cDNA amplification kit was used to prepare a cDNA library from one hour adherent macrophage. A 1040bp PCR fragment, isolated by PCR with sense and antisense oligonucleotide primers from the determined cDNA sequence were used, and the universal primer from the kit. This resulted in the isolation of a fragment of a larger size than the original 1040bp fragment. The new fragment which was sequenced added 60bp to the 5'sequence (SEQ ID NO:1).

RNA were extracted from mouse thioglycolate induced peritoneal macrophages after 1 hour adherence. Hybridization was performed with ³²P-labeled mTRELL cDNA. Figure 3 depicts northern analysis of TRELL mRNA expression in mouse peritoneal macrophages and in different mouse tissues.

The first 21 amino acids delineate a hydrophobic, transmembrane domain. No identical sequences at the nucleotide or the amino acid levels were found in the available databases. Using the PROSITE program, and the 225 amino acid sequence it was determined that the sequence belonged to the TNF family of proteins. The protein also possessed the different domains described for LT- α and other members of this family (J. Browning et al., Lymphotoxin- α , a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface", Cell, 72, 847 (1993); C. F. Ware et al., "The ligands and receptors of the lymphotoxin system", in Pathways for cytolysis, G. M. Griffiths and J. Tschopp (Eds), Springer-Verlag, Berlin, Heidelberg, p175-218 (1995), each of which is specifically incorporated herein by reference). This sequence is unique. At the nucleotide or amino acid levels, weak identity or similarity were observed with the different members of the TNF family or with any sequences. Searching in EST data bases, 1 human sequence was clearly homologous to the murine sequence. The clone 154742,5' (GenBank accession no: R55379) from a breast library made by Soares, Washington University, has a 345 base pair sequence, 89% homologous to the murine TRELL. No human sequence in the available databases was found matching the available 5' DNA of mTRELL.

b) Cloning of Human TRELL

i) Generation of oligonucleotide probes and PCR primers.

The sequence of the human EST R55379 which has homology to mouse TRELL was used as a basis for synthesis of oligonucleotide primers. Two sense strand

5 20mer oligonucleotides:

B LTB-065 5=-CCC TGC GCT GCC TGG AGG AA (NT 70-89 of R55379) (SEQ ID NO: 7)

B LTB-066 5=-TGA TGA GGG GAA GGC TGT CT (NT 14-33 of R55379) and one (SEQ ID NO: 8)

antisense 20mer oligonucleotide:

B LTB-067 5=-AGA CCA GGG CCC CTC AGT GA (NT 251- 270 of R55379) were (SEQ ID NO: 9)

10 synthesized.

ii) Identification of mRNA and cDNA library source for cloning hTRELL.

PolyA+ mRNA from Human liver (cat#6510-1), spleen (cat#6542-1) and lymph node (cat# 6594-1) were purchased from Clontech. PolyA+ mRNA from Human cell lines THP-1, U937 and II-23 were generated at Biogen, Cambridge, MA. A Human tonsil cDNA library in Lambda gt10, and DNA from the Tonsil library were also

15 prepared at Biogen.

RT-PCR was performed on the six RNA samples. Each cDNA reaction contained 1ug polyA+ mRNA, 50mM Tris pH 8.3, 75mM KCl, 3mM MgCl₂, 10 mM DTT, 250 uM dNTP, 50ng random hexamer (50ng/ul) and 400 units SuperscriptII

20 Reverse transcriptase (Gibco BRL cat#6542-1) in a final volume of 40 ul. The reaction was incubated at 20EC for 20 minutes, 42EC for 50 min., and 99EC for 5 min. For PCR, one-fifth of each cDNA reaction or 100-1000ng of the cDNA library DNA was used. Two PCR reactions for each sample were set up, one with primer pair LTB-065 and LTB-067 which yields a 201bp PCR product, and the second reaction with primer

25 pair LTB-066 and LTB-067 which yields a 257bp product if the transcript is represented in the sample. PCR reactions were performed in 10mM Tris pH8.3, 50mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 10% DMSO 100uM dNTP, 30pmole each primer and 5 units Amplitaq (Perkin Elmer cat#N801-0060). PCR was carried out in a Perkin Elmer Cetus DNA Thermal Cycler Model#480. Cycle conditions used were 95EC 1

30 minute, 60EC 1 minute, and 72EC 1 minute for 35 cycles.

The correct size products were obtained from liver, spleen, lymph node, THP-1 and tonsil, but not from U937 or II-23 mRNA. The 201bp PCR product generated from liver was purified for use as a probe for screening the cDNA library.

iii) cDNA Library Screening

Having demonstrated by PCR that the tonsil library contained TRELL, one million plaque forming units(PFU) from the Lambda gt10 Human tonsil cDNA library were plated at a density of 1×10^5 PFU / NuncTM plate. Duplicate lifts were made onto
5 20x20cm Schleicher and Scheull BA-S 85 OptitranTM filters. The 201bp PCR product was P³² labeled by random priming (Feinberg and Vogelstein, Anal. Biochem 137:266-267,1984 specifically incorporated herein by reference). The filters were hybridized overnight at 65EC in 400 ml plaque screen buffer (50mM Tris pH7.5, 1M NaCl, 0.1% sodium pyrophosphate, 0.2% PVP and 0.2% Ficoll) containing 10% dextran sulphate,
10 100ug/ml tRNA and 6×10^5 CPM/ml probe. They were washed twice with plaque screen buffer and twice with 2X SSC, 0.1% SDS at 65C and exposed to film at -70C with an intensifying screen for 40 hours.

Duplicate positives were cored from the master plates into SM (100mM NaCl, 10mM MgSO₄, 50mM Tris pH 7.5) plus gelatin. 12 of the positives were plaque
15 purified. Lambda miniprep DNA from 12 purified candidates was digested with Not1, electrophoresed on 1% agarose gel, Southern blotted and hybridized with the 201 bp probe. The clones with the largest inserts (approximately 2 kb) which hybridized to the probe were selected for large scale DNA purification and DNA sequencing. The inserts from each of these clones was subcloned into the Not1 site of pBluescript SK+
20 (Stratagene #212205). DNA sequence was obtained from the Lambda DNA and the plasmid DNA. Clone F1a which has an cDNA insert of 2006bp appeared to have an intron in the 5' end of the coding region and did not contain a complete open reading frame. Clone A2a, also called PB133 contained a cDNA insert of 1936bp. This clone contained 543bp 5' untranslated region, an open reading frame of 852bp and 3'
25 untranslated region but no polyadenylation signal or polyA tail.

The nucleotide sequence encoding the open reading frame of the hTRELL cDNA clone A2a is set forth in SEQ ID. NO. 3. The deduced 284 amino acid sequence is set forth in SEQ ID. NO. 4. The second methionine at position 36 may be a more likely translation start site, since this site more closely meets the requirements for a
30 start as defined by Kozak.

Using the sequences identified, the sequences of cDNAs coding on TRELL were determined. From the DNA sequences described above (i.e. SEQ. ID. NO. 3), we deduced the amino acid sequences of TRELL (SEQ. ID. NO. 4). It should be clear that given the current state of the protein-engineering art, an artisan could make purposeful

alterations, insertions or deletions in these amino acid sequences and obtain a variety of molecules having substantially the same biological or immunological activities as those of the molecules we have described herein.

iv. Northern Analysis of human TRELL expression

5 A 440 bp PpuM1/BstX1 fragment of the human cDNA clone 2a was 32P
labeled by random priming and used to probe commercial northern blots containing
RNA from various human tissues. Northern analysis showed that the hTRELL
fragment hybridized to a single mRNA species about 1.4 to 1.6 kb in length. Human
TRELL is expressed in most organs of the immune system, i.e. spleen, peripheral blood
10 lymphocytes (pbl), lymph nodes, appendix but was relatively low in thymus, fetal liver
(source of progenitor lymphocytes) and bone marrow (Figure 4). Therefore, organs of
the secondary immune system primarily express TRELL. Expression was also detected
in the ovary, prostate, small intestine, colon, heart, brain, placenta, lung, liver, skeletal
muscle, kidney and pancreas. Expression was relatively low in testis, liver, kidney and
15 thymus. This pattern indicates widespread expression closely resembling that of the
TRAIL ligand except that TRAIL is poorly expressed in heart and brain.

c) Isolation of a receptor binding to the TRELL Ligand

Ligands of the TNF family can be used to identify and clone receptors. With
the described TRELL sequence, one could fuse the 5' end of the extracellular domain of
20 TRELL ligand which constitutes the receptor binding sequence to a marker or tagging
sequence and then add a leader sequence that will force secretion of the ligand in any of
a number of expression systems. One example of this technology is described by
Browning et al., (1996) (JBC 271, 8618-8626) where the LT- β ligand was secreted in
such a form. The VCAM leader sequence was coupled to a short myc peptide tag
25 followed by the extracellular domain of the LT- β . The VCAM sequence is used to
force secretion of the normally membrane bound LT- β molecule. The secreted protein
retains a myc tag on the N-terminus which does not impair the ability to bind to a
receptor. Such a secreted protein can be expressed in either transiently transfected Cos
cells or a similar system, e.g., EBNA derived vectors, insect cell/baculovirus, picchia
30 etc. The unpurified cell supernatant can be used as a source of the tagged ligand.

Cells expressing the receptor can be identified by exposing them to the tagged
ligand. Cells with bound ligand are identified in a FACS experiment by labelling the

myc tag with an anti-myc peptide antibody (9E10) followed by phycoerythrin (or a similar label) labelled anti-mouse immunoglobulin. FACS positive cells can be readily identified and would serve as a source of RNA encoding for the receptor. An expression library would then be prepared from this RNA via standard techniques and separated into pools. Pools of clones would be transfected into a suitable host cell and binding of the tagged ligand to receptor positive transfected cells determined via microscopic examination, following labelling of bound myc peptide tag with an enzyme labelled anti-mouse Ig reagent, i.e. galactosidase, alkaline phosphatase or luciferase labelled antibody. Once a positive pool has been identified, the pool size would be reduced until the receptor encoding cDNA is identified. This procedure could be carried out with either the mouse ^{or} human TRELL' as one may more readily lead to a receptor.

2. Cells and Reagents

All cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) except for WEHI 164 clone 13 which was obtained from Dr. Kawashima (Geneva Biomedical Research Institute, Geneva, Switzerland). The HT29 subclone (HT29-14) was previously described (Browning et al., 1996) and the TNF sensitive ME180 subclone was obtained from Dr. Carl Ware. The II-23 T cell hybridoma has been described (Browning et al., 1991). Balb/c mice were injected intraperitoneally 3 days before sacrifice with 1.5 ml of thioglycolate broth (Difco Lab., MI). Cells were taken from the peritoneal cavity and cultured at 10^6 cells/ml for 1 hr in DMEM (Gibco Lab). Non adherent cells were washed off the plates and the adherent cells, almost exclusively macrophages, were lysed in Tri-Reagent (Molecular Research Center Inc.) and processed for RNA extraction.

Recombinant human TNF, LTa, LTa1/b2, antibodies to these proteins and the receptor-Ig fusion proteins have been described previously (Browning et al., 1995). The anti-CD40L antibody 5C8 has been described. A polyclonal anti-hTRELL serum was prepared by intra lymph node injection of pure recombinant hTRELL in CFA as described previously (Browning and Ribolini, 1989). After 2 months, an anti-hTRELL response was observed and immunoglobulin was purified using Protein A-Sepharose.

Mouse TRELL Cloning

The antisense oligonucleotide primer 5'GTTCCAGGCCAGCCTGGG3' from the mouse erythropoietin sequence was used in a 5' RACE protocol following the recommendation of the manufacturer (5' RACE system from BRL) in association with

50 ID NO: 10)

the BRL-designed anchor primer. First strand cDNA was made from RNA from 1 hr. adherent peritoneal macrophages. Amplification was done in a Perkin Elmer DNA thermal cycler with Taq DNA polymerase. After a denaturation of 5min. at 94/ C, cycling conditions were as follows: 35 cycles of 30 sec. at 94/ C, 30 sec at 55/ C and 3 min at 72/ C followed by a terminal additional extension at 72/ C. Analysis of the PCR experiment on agarose gel revealed 2 amplified fragments of 650 bp and 500 bp. The 2 fragments were excised from the gel, inserted in pBS-T vectors and sequenced. Northern hybridizations with ³²P labeled-random-primed fragments indicated that the 500 bp fragment hybridizing to a 1.4 kb RNA in macrophages. To determine the orientation of the cDNA, 32P-labeled-riboprobes in both direction were used in Northern hybridization. From the determined orientations and sequences, we derived two internal primers for the 1.4 kb mRNA: 5' TCAGGTGCACTTTGATGAGG 3' and 5'CTGTCAGCTCCTCCTGAG 3' which were used in 3' and 5'RACE-PCR respectively. The 3'RACE experiment revealed a 750bp fragment which was inserted in a pBS-T vector and sequenced. It corresponded to the 3' end of the 1.4 kb RNA since the sequence possessed a polyA addition signal just prior to the poly A tract. The 5'RACE did not revealed any band. The Clontech Marathon cDNA amplification kit was used to prepare a cDNA library from 1 hr. adherent macrophages. PCR used a 1040 bp PCR fragment isolated with sense and antisense oligonucleotide primers from the determined cDNA sequence (5'AGCAGGAGCCTTCTCAGGAG 3' and 5'GATCCAGGGAGGAGCTTGTC 3') and the universal primer from the kit. This resulted in the isolation of a fragment 60 bp longer on the 5' end than the original 1040 bp fragment.

Human TRELL Cloning

A search of the EST data base showed 1 human clone that was clearly homologous to the murine sequence. The clone 154742 (Genbank accession no: R55379) has a 345 bp sequence 89% homologous to the murine cDNA. Two primers derived from the EST (5' CCCTGCGCTGCCTGGAGGAA 3' and 5' AGACCAGGGCCCCCTCAGTGA 3') were used to screen by RT-PCR different tissues and libraries for the presence of hTRELL transcripts. Correct size products were obtained from liver, spleen, lymph node, THP-1 and tonsil, but not from U937 mRNA. The 201 bp product was cloned and used to screen a lambda gt10 human tonsil cDNA library. 10⁶ plaque forming units were plated at 10⁵ PFU/plate. Duplicate lifts were made onto 20x20 cm nitocellulose filters and hybridized with a probe prepared by

random-priming. The filters were hybridized overnight at 65/ C in plaque screen buffer (50 mM Tris pH7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.2% polyvinylpyrrolidone and 0.2% Ficoll) containing 10% dextran sulphate, 100 mg/ml tRNA and 6×10^5 cpm/ml of probe. They were washed twice with plaque screen buffer and twice with 2XSSC, 0.1% SDS at 65/ C. Lambda miniprep DNAs were prepared from positive colonies and the clones with the largest inserts were selected for large scale DNA purification and DNA sequencing. The inserts were subcloned into the NotI site of pBlueScript SK+.

One human EST (R55379) was found encoding parts of the human TRELL sequence.

10 RNA Analysis

Either a 0.45 kb PpuM1/BstX1 or a 1.25 NarI/NotI fragment of the hTRELL cDNA was labeled by random priming and used to probe human and mouse tissue northern blots purchased from Clontech. Mouse tissues and cells were RNA-extracted with TRI-reagent. Northern analysis were done essentially as already described (Chicheportiche and Vassalli, 1994) with 4 ug of total RNA and $32P$ labeled random primed mTRELL cDNA.

Chromosomal assignment

A panel of DNA from monochromosomal cell hybrids (HGMP Resource centre, Hinxton, Cambridge, UK) was used to amplify by PCR a 340bp fragment with primers chosen in 3' untranslated region that are not homologous to the murine sequence (5' AGTCGTCCCAGGCTGCCGGCT 3' and 5' CCTGAAGTGGGGTCTTCTGGA 3'). Amplification was done for 40 cycles, 30 sec at 94/ C, 90 sec at 65/ C and 90 sec at 72/ C. Detection was carried out on ethidium bromide stained agarose gel.

Expression of Recombinant hTRELL Protein

A soluble expression construct combining the VCAM leader sequence, the myc peptide tag and the extracellular domain of hTRELL similar to that described for lymphotoxin-b (ref) was prepared in a manner similar to that described for LTb (Browning et al., 1996). The following DNA fragments were isolated, a NotI/blunt fragment encoding the VCAM leader and a pair of oligonucleotides encoding the myc tag (5' blunt, 3' PpuM1 site) which have been described, a 0.45 kb PpuM1/BstX1 fragment of TRELL and a 0.65 BstX1/NotI fragment of TRELL. The four fragments were ligated into a NotI/phosphatased pBluescript vector. The NotI insert from this vector was transferred into the pFastBac1 vector (GibcoBRL) and used to generate

recombinant baculovirus. Soluble TRELL was prepared by infecting HiFiveTM insect cells at a MOI of 10 and the medium was harvested after 2 days. The following items were added to the media : HEPES buffer to a final concentration of 25 mM, pH 7.4, 1 mM AEBSF (Pierce) and 1 mg/ml pepstatin. The media was filtered and concentrated ten fold by ultrafiltration over a Amicon 10 kDa cutoff filter. Concentrated TRELL containing medium was directly loaded onto a SP sepharose Fast Flow column and washed with 25 mM HEPES buffer pH 7.0 containing 0.4 M NaCl. TRELL was eluted with the same buffer with 0.6 M NaCl. Purified TRELL was subjected to sizing analysis ~~on a~~ ^{by gel exclusion chromatography.}

10 **Analysis of Secretion**

Vectors for EBNA based expression were constructed using the vector CH269 which is a modified version of the pEBVHis ABC (Invitrogen) wherein the EBNA gene and the histidine tag were removed. A 0.71 kb fragment of hTNF in the pFastBac vector was provided by Dr. P. Pescamento and A. Goldfeld. The SnaBI/XhoI insert was ligated into the PvuII/XhoI site of CH269. A genomic TNF insert containing the _1-12 cleavage site deletion was a gift from Dr. G. Kollias and was inserted into the CH269 vector by A. Goldfeld. A 1.8 kb NotI insert of hTRELL clone A2A, the 0.98 kb NotI fragment containing the hCD40L cDNA provided by Dr. E. Garber and a 1.46 kb NotI insert containing hLTa (Browning et al., 1995) were ligated into the NotI site of CH269. A 0.81 kb HindIII insert containing the hLTb coding region with a modified start site (Browning et al., 1995) was ligated into the HindIII site of CH269. EBNA-293 cells were transfected with the various CH269 vectors along with the GFP vector using lipofectamine and either removed with PBS with 5 mM EDTA for FACS analysis or after 2 days the cells were subjected to metabolic labelling. Both procedures utilized the following antibodies, hTRELL a rabbit polyclonal Ig fraction, hTNF the mAb 104c, hLTa the mAb AG9, LTa1/b2 the mAb B9 and CD40L the mAb 5C8. FACS analysis was carried out in RPMI medium containing 10% FBS and 50 ug/ml heat aggregated human IgG with the antibodies at 5 ug/ml. Phycoerythrin labelled anti-mouse or rabbit IgG (Jackson ImmunoResearch) was used to detect antibody binding. GFP bright transfected cells were live gated. For immunoprecipitation, cells 2 days after transfection were washed with PBS and transferred into met/cys free MEM containing 200 uCi/ml TranSlabel (ICN). After 3 h the supernatants were harvested and subjected to immunoprecipitation as described (Browning et al., 1995).

Cytotoxicity Assays:

Cell growth assays were carried out as previously described (Browning and Ribolini, 1989). For microscopy, HT29-14 cells were seeded into 12 well plates at a density of 200,000 cells/well and grown for 2 days. Human TRELL, TNF, lymphotoxin-a1b2 (Browning et al., 1996) or anti-fas (CH11, Kamaya) were added along with 80 units/ml of human interferon-g. After 26 h, the medium was removed which after cytokine or anti-fas treatment included many dead cells that had detached from the plastic. The remaining cells were fixed with 80% ethanol and washed into PBS containing 1 mg/ml Hoescht dye. After 2 min the dye was removed, cells were washed into PBS and examined by fluorescence microscopy.

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Table II: Human TRELL Binding Sites and Cytotoxic Effects on Various Cell Lines

	<u>Cell Line</u>	<u>Type</u>	<u>TRELL Binding</u>	<u>Cytotoxicity^a</u>
5	<u>Hematopoietic</u>			
	Jurkat	T lymphoma	-	-
10	SKW 6.4	EBV B cell	-	-
	Namalwa	Burkitt lymphoma	-	-
	K562	promyelocytic	+	-
	THP-1	monocytic leukemia	++	-
15	<u>Nonhematopoietic</u>			
	HT29	colon adenocarcinoma	+	++ ^b
	ME-180	cervical carcinoma		-
	Hela	cervical carcinoma		- ^d
	MCF-7	breast adenocarcinoma		+/-
20	293	embryonic kidney cells	+	nd
	Cos	kidney fibroblasts	+	nd

25 ^{B?} ^a3-5 day proliferation assay in the presence and absence of human interferon-g.

^bCytotoxicity was only observed in the presence of interferon-g.

^cND, not determined.

^dMorphology changes

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Table III: Grouping of Various TNF Family Members by Cytotoxicity Patterns

	<u>Group</u>	<u>Receptor Activation</u>
5	Potent inducers of apoptosis in many cell types	TNF, Fas, TRAIL-R ^a , DR-3
	Weak inducers only in limited cell types	LTb-R, TRELL-R ^a , CD30
10	Cannot induce cell death, anti-proliferative in some settings	CD27, CD40, OX-40

15 ^aThese receptors have not yet been identified.

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- i. Smith et al. 1990; Kohno et al 1990; Loetscher et al 1990; Schall et al 1990.
 - ii. See Jones et al., 1989; Eck et al., 1992.
 - iii. K. Tracey, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 255 (1992)); A. Waage, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 275 (1992).
 - iv. G. D. Roodman, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 117 (1992).
 - v. A. Nakane, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 285 (1992); I. A. Clark et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 303 (1992); G. E. Grau et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 329 (1992); P-F. Piguet, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 341 (1992); G. H. Wong et al., in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 371 (1992).
 - vi. S. Malik, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 407 (1992).
 - vii. D. A. Fox, Am. J. Med., 99, 82 (1995).
 - viii. D. Goeddel et al., Cold Spring Harbor Symposium Quant. Biol., 51, 597 (1986); G. Trinchieri, in Tumor Necrosis Factors. The Molecules and Their Emerging Role in Medicine, B. Beutler (Ed.), Raven Press, NY, p 515 (1992).

-
- ix. L. A. Tartaglia et al., Proc. Natl. Acad. Sci. USA, 88, 9292 (1991); L.A. Tartaglia and D. V. Goeddel, Immunol. Today, 13, 151 (1992).
- x. B. Luetting et al., J. Immunol., 143, 4034 (1989); M. Kriegler et al., Cell, 53, 45 (1988).
- xi. C. F. Ware et al., in Pathways for Cytolysis, G. M. Griffiths and J. Tschopp (Eds.), Springer-Verlag, Berlin, Heidelberg, p175-218 (1995).
- xii. N. Paul et al., Ann. Rev. Immunol., 6, 407 (1988).
- xiii. P.D. Crowe et al., Science, 264, 707 (1994). (J. Browning et al., Cell, 72, 847 (1993); J. Browning et al., J. Immunol., 154, 33 (1995).
- xiv. P. De Togni et al., Science, 264, 703 (1993); T.A. Banks et al., J. Immunol., 155, 1685 (1995).
- xv. J. Browning and A. Ribolini, J. Immunol., 143, 1859 (1989); J. Browning et al., J. Exp. Med., 183, 867 (1996).
- xvi. T. Suda et al., J. Immunol., 154, 3806 (1995); (T. Suda et al., J. Immunol., 154, 3806 (1995).
- xvii. B.C. Trauth et al., Science, 245, 301 (1989); S. Yonehara et al., J. Exp. Med., 169, 1747 (1989); S. Nagata and P. Goldstein, Science, 267, 1449 (1995); M. H. Falk et al., Blood, 79, 3300 (1992).
- xviii. F. Rieux-Laucat et al., Science, 268, 1347 (1995); T. Takahashi et al., Cell, 76, 969 (1994); R. Watanabe-Fukunaga et al., Nature, 356, 314 (1992).
- xix. P. R. Galle and al., J. Exp. Med., 182, 1223 (1995).
- xx. F. Silvestris and al., Clin. Immunol. Immunopathol., 75, 197 (1995).
- xxi. P.D. Katsikis et al., J. Exp. Med., 181, 2029 (1995); A. D. Badley et al., J. Virol., 70, 199 (1996).
- xxii. S. Wiley et al., Immunity, 3, 673 (1995).
- xxiii. J. F. Gauchat et al., FEBS Lett., 315, 259 (1993); S. Funakoshi et al., Blood, 83, 2787 (1994).
- xxiv. R. C. Allen et al., Science, 259, 990 (1993).
- xxv. L. Biancone et al., Kidney-Int., 48, 458 (1995); C. Mohan et al., J. Immunol., 154, 1470 (1995).
- xxvi. J. Ruby and al., Nature Medicine, 1, 437 (1995).

-
- xxvii. Z. Wang et al., J. Immunol., 155, 3722 (1995); A. M. Cleary and al., J. Immunol., 155, 3329 (1995).
- xxviii. S. Hess and H. Engelman, J. Exp. Med., 183, 159 (1996).
- xxix. R. G. Goodwin et al, Cell, 73, 447 (1993); Goodwin et al, Eur. J. Immunol., 23, 2631 (1993); C. A. Smith et al., Cell, 73, 1349 (1993).
- xxx. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover ed., 1985); Oligonucleotide Synthesis (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); Transcription And Translation (B. D. Hames & S. J. Higgins eds. 1984); Culture Of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al. eds.), Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).
- xxxi. See for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477.
- xxxii. See, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) PNAS 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) PNAS 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815.
33. M.T. Abreu-Martin, A. Vidrich, D.H. Lynch and S.R. Targan. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNF- α and ligation of Fas ligand. J. Immunol. 155: 4147-4154, 1995.
34. K. Agematsu, T. Kobata, F.-C. Yang, T. Nakazawa, K. Fukushima, M. Kitahara, T. Mori, K. Sugita, C. Morimoto and A. Komiyama. CD27/CD70 interaction directly drives B cell IgG and IgM synthesis. Eur. J. Immunol. 25: 2825-2829, 1995.
35. R. Amakawa, A. Hakem, T.M. Kundig, T. Matsuyama, J.J.L. Simard, E. Timms, A. Wakeham, H.-W. Mittrucker, H. Griesser, H. Takimoto, R. Schmits, A. Shahinian, P.S. Ohashi, J.M. Penninger and T.W. Mak. Impaired negative selection of T cells in Hodgkin's disease antigen CD30-deficient mice. Cell 84: 551-562, 1996.

36. J.-L. Bodmer, K. Burens, P. Schneider, K. Hofmann, V. Steiner, M. Thome, T. Bornand, M. Hahne, M. Schroeter, K. Becker, A. Wilson, L.E. French, J.L. Browning, H.R. MacDonald, and J. Tschopp. TRAMP, a novel apoptosis-mediating receptor with sequence homology to tumor necrosis factor receptor 1 and fas (apo-1/CD95). Immunity 6: 79-88, 1997.
37. J. Brojatsch, J. Naughton, M.M. Rolls, K. Ziegler and J.A.T. Young. Carl, a TNFR-related protein is a cellular receptor for cytopathic avian leukemia-sarcoma viruses and mediates apoptosis. Cell 87: 845-855, 1996.
38. J.L. Browning, M.J. Androlewicz and C.F. Ware. Lymphotoxin and an associated 33-kDa glycoprotein are expressed on the surface of an activated human T cell hybridoma. J. Immunol. 147: 1230-7, 1991.
39. J.L. Browning, K. Miatkowski, D.A. Griffiths, P.R. Bourdon, C. Hession, C.M. Ambrose and W. Meier. Preparation and characterization of soluble recombinant heterotrimeric complexes of human lymphotoxins alpha and beta. J. Biol. Chem. 271: 8618-26, 1996.
40. J.E. Castro, J.A. Listman, B.A. Jacobson, Y. Wang, P.A. Lopez, S. Ju, P.W. Finn and D.L. Perkins. Fas Modulation of apoptosis during negative selection of thymocytes. Immunity 5: 617-627, 1996.
41. C.-Y.A. Chen and A.-B. Shyu. AU-rich elements: characterization and importance in mRNA degradation. Trends in Biol. Sci. 20: 465-470, 1995.
42. Y. Chicheportiche, C. Ody and P. Vassalli. Identification in mouse macrophages of a new 4 kb mRNA present in hematopoietic tissue which shares a short nucleotide sequence with erythropoietin mRNA. Biochim. Biophys. Res. Comm. 209: 1076-1081, 1995.
43. A.M. Chinnaiyan, K. O'Rourke, G.-L. Yu, R.H. Lyons, M. Garg, D.R. Duan, L. Xing, R. Gentz, J. Ni and V.M. Dixit. Signal transduction by DR3 a death-domain-containing receptor related to TNFR-1 and CD95. Science 274: 990-992, 1996.
44. P. DeTogni et al. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 264: 703-7, 1994.
45. M.A. DeBenedette, N.R. Chu, K.E. Pollok, J. Hurtako, W.F. Wade, B.S. Kwon and T.H. Watts. Role of 4-1BB ligand in costimulation of T lymphocyte growth and its upregulation on M12 B lymphomas by cAMP. J. Exp. Med. 181: 985-992, 1995.
46. M. Degli-Esposti, T. Davis-Smith, W.S. Din, P.J. Smolak, R.G. Goodwin and C.A. Smith. Activation of the lymphotoxin- β receptor by cross-linking induces chemokine production and growth arrest in A375 melanoma cells. J. Immunol. 158: 1756-1762, 1997.
47. T.M. Foy, A. Aruffo, J. Bajorath, J.E. Buhlmann and R.J. Noelle. Immune regulation by CD40 and its ligand gp39. Ann. Rev. Immunol. 14: 591-617, 1996.

-
48. H.J. Gruss, N. Boiani, D.E. Williams, R.J. Armitage, C.A. Smith and R.G. Goodwin. Pleiotropic effects of the CD30 ligand on CD30-expressing cells and lymphoma cell lines. Blood 83: 2045-56, 1994.
 49. H.J. Gruss and S.K. Dower. Tumor necrosis factor ligand superfamily: involvement in the pathology of malignant lymphomas. Blood 85: 3378-404, 1995.
 50. J. Kitson, T. Raven, Y.-P. Jiang, D.V. Goeddel, K.M. Giles, K.-T. Pun, C.J. Grinham, R. Brown and S.N. Farrow. A death domain-containing receptor that mediates apoptosis. Nature 384: 372-375, 1996.
 51. S.Y. Lee, C.G. Park and Y. Choi. T cell receptor-dependent cell death of T cell hybridomas mediated by the CD30 cytoplasmic domain in association with tumor necrosis factor receptor-associated factors. J. Exp. Med. 183: 669-674, 1996.
 52. R.I. Montgomery, M.S. Warner, B.J. Lum and P.G. Spear. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87: 427-436, 1996.
 53. S. Nagata. Apoptosis by death factor. Cell 88: 355-365, 1997.
 54. R.M. Pitti, S.A. Marsters, S. Ruppert, C.J. Donahue, A. Moore and A. Ashkenazi. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. 1996.
 55. C.A. Smith, T. Farrah and R.G. Goodwin. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76: 959-62, 1994.
 56. G.L. Smith. Virus strategies for evasion of the host response to infection. Trends in Microbiol. 3: 81-88, 1994.
 57. E. Strueber and W. Strober. The T cell-B cell interaction via OX40-OX40L is necessary for the T cell independent humoral immune response. J. Exp. Med. 183: 979-989, 1996.
 58. H.-K. Sytwu, R.S. Liblau and H.O. McDevitt. The roles of Fas/Apo-1 (CD95) and TNF in antigen-induced programmed cell death in T cell receptor transgenic mice. Immunity 5: 17-30, 1996.
 59. P. Vassalli. The pathophysiology of tumor necrosis factors. Ann. Rev. Immunol. 10: 411-452, 1992.
 60. L. Zheng, G. Fisher, R.E. Miller, J. Peschon, D.H. Lynch and M.J. Lenardo. Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377: 348-351, 1995.